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Pharmacological Evaluation of the Ethanolic Leaf Extract of *Stephania japonica*: Evidence of Cytotoxicity, Antidiabetic Effects, and Analgesic Potential

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ABSTRACT

Background: *Stephania japonica* is a medicinal plant utilized in many folk medicines and is reported to contain bioactive phytochemicals with therapeutic properties. The aim of the present study was to evaluate the cytotoxic, antidiabetic, and analgesic properties of the ethanolic leaf extract of *Stephania japonica*.

Methods: Cytotoxic effect of the extract was evaluated by the brine shrimp lethality bioassay where vincristine sulfate was considered as the reference compound. Antidiabetic activity was evaluated in glucose loaded mice using the Oral Glucose Tolerance Test (OGTT) and metformin (100 mg/kg) was employed as the standard drug. Analgesic activity was evaluated in the acetic acid-induced writhing test in Swiss albino mice, while diclofenac sodium (100 mg/kg) was employed as the standard analgesic drug.

Results: In the brine shrimp lethality bioassay, the ethanolic leaf extract of *Stephania japonica* showed a concentration-dependent cytotoxic activity with an LC_{50} value of 31.74 µg/mL, while the vincristine sulfate displayed an LC_{50} value of 1.07 µg/mL. In the OGTT model, the leaf extract at 500 mg/kg dose significantly lowered the plasma glucose level, causing 75.13% reduction after 240 minutes as compared with 84.41% caused by metformin. In the analgesic study, the ethanolic leaf extract at 500 mg/kg caused reduction in the number of writhes from 45 in the control group to 26, i.e., 42.22% inhibition of writhing while diclofenac sodium produced 91.11% inhibition.

Conclusion: It can be concluded that the ethanolic leaf extract of *Stephania japonica* displays considerable cytotoxic effect and antihyperglycemic effect and moderate analgesic effect.

Keywords: Analgesic Activity; Antidiabetic Activity; Brine Shrimp Lethality Assay; Cytotoxicity; Ethanolic Leaf Extract; Glucose Tolerance Test; Medicinal Plants; Pharmacological Evaluation; *Stephania japonica*.

1.0. Introduction

Stephania japonica, a climber belonging to Menispermaceae, has been found to occur extensively in South and Southeast Asia including Bangladesh, India, China and Malaysia. The herb has been used in traditional medicine for the treatment of many diseases such as fever, inflammation, pain, digestive problems and metabolic disorders [1]. Genus Stephania has been documented to consist of many phytoconstituents such as alkaloids, flavonoids, terpenoids, glycosides and phenols with marked pharmacological activities [2-4]. Various biological activities such as antioxidant, antimicrobial, anti-inflammatory, anticancer, antidiabetic and analgesic have been identified in various Stephania species previously reported [5-6].

Assessment of cytotoxic activity is an important initial stage for the detection of bioactive compounds having potential anticancer and pesticidal activities. Brine shrimp lethality assay is a very simple, fast and inexpensive technique that has been used to determine the cytotoxic activity of various plant extracts [7-8]. Correlation has been noted between brine shrimp lethality and other pharmacological activities such as antitumor and antimicrobial activities. Thus, determination of cytotoxic activity may offer useful information regarding the presence of bioactive compounds in medicinal plants [9].

Diabetes mellitus is a chronic metabolic disorder in which there is continuous presence of hyperglycemia due to impairment in the functioning of insulin. The prevalence of diabetes mellitus around the world has escalated significantly in recent years causing major health concern. Continuous hyperglycemia may cause serious complications such as cardiovascular, renal, neurological and ocular disorders. Though there are a number of antidiabetic drugs available but many of them suffer from side effects and high cost [10]. Hence, there is much interest to find new and more effective antidiabetic agents from medicinal plants. Assessment of antihyperglycemic activity through Oral Glucose Tolerance Test (OGTT) is an efficient method to assess the hypoglycemic property of plant extracts [11].

Pain is one of the most prevalent symptoms of many diseases and has a huge impact on quality of life. Nonsteroidal anti-inflammatory drugs (NSAIDs) and opioids are among the major therapeutic drugs used for managing pain; but prolonged usage of these drugs causes a number of side-effects such as stomach irritation, kidney problems, tolerance and addiction [4]. Thus, there have been a lot of efforts for exploring alternative pain relievers from natural resources. Acetic acid induced writhing test is a well-known method of testing peripheral analgesic activity of the compound and is widely used for pharmacological evaluation of medicinal plants [11].

Even though the traditional application of *Stephania japonica* is well known and some of the activities of the other species in this genus have been documented scientifically, there is not much scientific data about cytotoxicity, antidiabetic activity and analgesic activity of the leaves of *Stephania japonica*. Thus, the current study was designed to evaluate the cytotoxic activity of the ethanolic leaf extract of *Stephania japonica* using the brine shrimp lethality assay, to test the antidiabetic activity using OGTT in mice and to check the analgesic activity using acetic acid induced writhing test. The results of this study will help validate the traditional medical use of *Stephania japonica* scientifically and lay a basis for further research to isolate and characterize active compounds.

1.1. Study Objectives

The overall goal is to study the cytotoxicity, anti-diabetic and analgesic properties of the ethanolic leaf extract of *Stephania japonica* using the in vitro and in vivo animal experimentation models. The specific goals are as follows:

1) To conduct the bio-assay for measuring the cytotoxic property of the ethanolic leaf extract of *Stephania japonica* using the brine shrimp lethality test.

2) To measure the median lethal concentration (LC_{50}) of the extract and to compare the cytotoxicity of the extract with the standard drug vincristine sulfate.

3) To carry out the anti-diabetic activity of the ethanolic leaf extract of *Stephania japonica* in glucose loaded mice using the Oral Glucose Tolerance Test (OGTT).

4) To compare the anti-hyperglycemic activity of the extract with the standard anti-diabetic drug metformin.

5) To investigate the analgesic activity of the ethanolic leaf extract using the acetic acid-induced writhing test in Swiss albino mice.

6) To compare the analgesic activity of the extract with the standard analgesic drug diclofenac sodium.

7) To validate the traditional medicinal value of *Stephania japonica*.

8) To build a foundation for further studies on phytochemicals and pharmacology of isolation and characterization of bioactive components of *Stephania japonica*.

2.0. Literature Review

Systematic studies on the Stephania species have shown antimicrobial, antiviral, antitumor, antioxidant, antihyperglycemic, anti-inflammatory, antinociceptive, neuroprotective, cardioprotective and immunomodulatory effects. The pharmacological activities of Stephania are primarily due to the alkaloids present in the plant with diversified structures [1].

Nevertheless, natural products still represent one of the major sources of anticancer drugs discovery. Many of the currently available anticancer medicines such as vincristine, vinblastine, paclitaxel, and camptothecin analogues were discovered from plants [12]. Hence, evaluation of the cytotoxic activity of plant extracts represents a necessary step in the search for potentially active anticancer compounds [7]. Brine shrimp lethality bioassay is commonly used in the process of cytotoxic activity estimation because of its simplicity, inexpensiveness, and reliability. The bioassay represents the assessment of test compound activity in the induction of the death of *Artemia salina* larvae. Research has proven the existence of a positive relationship between the brine shrimp lethality and such properties as antitumor, pesticide, antiviral, and antimicrobial actions [8]. A number of medicinal plants from the Menispermaceae family have shown considerable cytotoxic activity. It is thought that the presence of alkaloid and phenolic compounds in these plants contributes to their ability to kill cancer cells. Thus, evaluation of the cytotoxic activity of *Stephania japonica* could be helpful in understanding its possible medical use [9].

Diabetes mellitus is a chronic metabolic condition caused by high blood glucose levels due to inadequate secretion of insulin or insulin resistance. Diabetes has emerged as one of the main health problems all over the world since hundreds of millions of people suffer from this disease. Complications associated with diabetes include cardiovascular disorders, nephropathy, neuropathy, and retinopathy. Medicinal plants have been extensively studied as alternative medicines for the treatment of diabetes. Active components found in medicinal plants may exhibit antihyperglycemic properties through various mechanisms such as insulin secretion stimulation, glucose uptake increase, carbohydrate digestive enzyme inhibition, and oxidation decrease [10]. The Oral Glucose Tolerance Test (OGTT) is used in the research aimed at determination of antidiabetic properties of medicinal plant extracts. Many studies report on pronounced glucose-lowering properties of alkaloid-containing and flavonoid-containing plant extracts. As *Stephania japonica* possesses such classes of phytochemicals, the herb can be potentially effective in the treatment of diabetes [11].

The use of medicinal plants as natural medicines for pain management dates back centuries. Various bioactive compounds such as flavonoids, alkaloids, tannins, and terpenoids have been found to play roles in the regulation of the perception of pain through inhibition of the biosynthesis and secretion of inflammatory mediators, mainly

prostaglandins [12]. One of the most commonly used methods for investigating peripheral antinociceptive activity is the acetic acid-induced writhing method. Acetic acid causes the release of endogenous mediators like prostaglandins, histamine and serotonin, resulting in characteristic writhing behavior in mice. Decrease in the number of writhes indicates antinociceptive activity. Various medicinal plants rich in alkaloids have shown good antinociceptive effects in this method. This suggests that *Stephania japonica* may have similar pharmacological activity as well [4].

This study has thus been conducted to explore the cytotoxic activity of the ethanolic leaf extract of *Stephania japonica* by the use of the brine shrimp lethality assay, its antidiabetic effect by the use of Oral Glucose Tolerance Test (OGTT) and its analgesic activity by using the acetic acid-induced writhing method in Swiss albino mice.

3.0. Materials and Methods

3.1. Chemicals and Reagents

Analytical grade chemicals and reagents were used in the current investigation. Ethanol was used as the solvent for extraction of the plant. Vincristine sulfate served as the standard drug in the brine shrimp lethality bioassay. Metformin hydrochloride served as the reference antidiabetic drug in the Oral Glucose Tolerance Test (OGTT) while diclofenac sodium served as the standard analgesic agent in the acetic acid-induced writhing test. Other chemicals used in the experiment included glucose, sodium chloride, dimethyl sulfoxide (DMSO), artificial seawater, and glacial acetic acid [7].

3.2. Experimental Animals

Swiss albino mice of either sex, weighing 25-35 g, were used for the antidiabetic and analgesic tests. The animals were purchased from an approved animal breeding laboratory and maintained in standard laboratory conditions of constant temperature, humidity, and 12-hour day/night cycles. The animals had ad libitum access to standard pellets and tap water. One week before initiation of experimentation, the animals were acclimatized to laboratory conditions [1].

3.3. Brine Shrimp Nauplii

Eggs of brine shrimp (*Artemia salina*) were used in the cytotoxicity assay. The eggs were hatched in artificial seawater under constant aeration and illumination for 24-48 hours to obtain live nauplii for the bioassay [8].

3.4. Preparation of Crude Extract (Cold Extraction Method)

Leaves of *Stephania japonica* were collected fresh from the area of Chandpur, Chittagong, Bangladesh. Plant material was identified and authenticated by a botanist. The leaves were cleaned with distilled water for dusting and other contaminants. Afterwards, the leaves were dried under the shade at room temperature until they attained a uniform weight. After drying, the leaves were grinded into a coarse powder using a mechanical grinder. The leaves (430 grams) were then added to 1200 mL ethanol in a clean, flat-bottomed glass vessel. This was sealed and allowed to remain for 12 days under room temperature with occasional shaking of the leaves to help in the extraction process. The solution was filtered through a clean cotton cloth to remove any foreign materials from it

and later filtered using Whatman No. 1 filter paper. This solution is filtered clean with only one filter paper. The solvent evaporation was carried out in a rotary evaporator at reduced pressure to obtain dry crude extract which was stored at 4^{o}C in a glass bottle [1,13].

3.5. Lethal Brine Shrimp Bioassay

To determine the cytotoxicity potential of the ethanolic leaf extract of *Stephania japonica*, the brine shrimp lethality bioassay test was employed following the protocol by Meyer et al. The eggs of the brine shrimps (*Artemia salina*) were hatched in artificial seawater with constant aeration and illumination for 24–48 hours. Serial dilution was done to prepare various concentrations of the plant extract from 0.078 to 160 µg/mL. Each of the test tubes contained 10 active nauplii which were transferred in different concentrations of the extract. The vincristine sulfate was the positive control while the artificial seawater was the negative control. The survival nauplii count after 24 hours of incubation was measured and the percentage mortality was calculated using the formula below:

$$\text{Mortality (\%)} = \frac{\text{Number of dead nauplii}}{\text{Total number of nauplii}} \times 100 \qquad (1)$$

The LC_{50} was calculated based on the percentage mortality vs. log of concentration graph [9].

3.6. Oral Glucose Tolerance Test (OGTT)

In the evaluation of the antidiabetic properties of the ethanolic leaf extract, Oral Glucose Tolerance Test (OGTT) on glucose loaded mice was used. Fasting was done for 16 hours for all the animals used in the experiment with the provision of water. Three groups of mice (n = 3) were selected as Group I (Control): Distilled water (10 mL/kg), Group II (Standard): Metformin hydrochloride (100 mg/kg) and Group III (Test): Ethanolic leaf extract of *Stephania japonica* (500 mg/kg). After 30 minutes of administration of the drugs, the glucose solution was given to all the groups. Collection of blood samples was done in five different time intervals (0, 60, 120, 180, 240 min). Plasma glucose level was determined using the glucometer. The percentage reduction in plasma glucose level was calculated and compared among the groups [11].

3.7. Analgesic activity

A writhing test was conducted in the mouse model using acetic acid, with the objective of evaluating the analgesic effect of the plant extracts. Five mice were taken in each of the three randomly selected groups. The first group served as the control group and was given 10 mL per kg of body weight using distilled water. The standard drug Diclofenac Sodium was administered at the dose of 100 mg/kg body weight in group two. In group three, 500 mg/kg body weight of the extract was administered. The experimental extract, drug and control solvent were administered orally 30 minutes before intraperitoneally injecting 0.7% v/v acetic acid. Five minutes later, the number of writhes was counted after 15 minutes [11]. The percent writhing inhibition obtained in the experimental and standard treatments was then compared to the writhing mean of the control treatment using this formula:

$$\text{Writhing inhibition (\%)} = 1 - \frac{\text{Writhing mean of control}}{\text{Writhing mean of sample/standard}} \times 100 \qquad (2)$$

4.0. Results and Discussion

The current study tested the cytotoxic, antidiabetic, and analgesic activities of the ethanolic leaf extract of *Stephania japonica* using standardized in vitro and in vivo models. It has been found that the extract is endowed with significant biological activities, which support its usage in traditional medicine and the presence of bioactive phytochemicals in the plant.

4.1. Cytotoxic Activity

The cytotoxicity of the ethanolic leaf extract of *Stephania japonica* was evaluated using brine shrimp lethality bioassay test, one of the most widespread assays to screen biologically active substances. As shown in Table 1 and Figure 1, the extract showed the lethal effect in a dose-dependent manner on *Artemia salina* larvae, causing death of 10% at the concentration of 5 µg/mL up to 90% mortality at 160 µg/mL, thus revealing the significant dose-dependent relationship. The extract revealed the LC_{50} of 31.74 µg/mL, calculated using Equation 1, while the reference substance vincristine sulfate revealed a significantly lower LC_{50} of 1.07 µg/mL.

Despite the difference in the cytotoxic potency between the extract and vincristine sulfate, the LC_{50} value shows the presence of significant cytotoxic components. According to the literature data, species belonging to the genus of Stephania contain numerous alkaloids, especially bisbenzylisoquinoline alkaloids, which are characterized by cytotoxic and antitumor activity. Hence, the cytotoxic activity detected in the current study might be explained by the presence of these phytochemicals. Similar results were obtained for other medicinal plants containing alkaloids and phenols, where significant brine shrimp lethality was associated with the anticancer activity [14].

Table 1. Brine Shrimp lethality bioassay of ethanolic leaves extract of *Stephania japonica* (Vincristine Sulfate as Standard).

Concentration (µg/mL)	**Log C**	**Alive Nauplii (n = 10)**			**Mortality (%)**		**LC_{50} (µg/mL)**
		Control	Standard	Extract	Standard	Extract	
0.078	-1.108	10	9	-	10	-	Vincristine Sulfate Standard (1.07)
0.156	-0.807	10	8	-	20	-	
0.313	-0.504	10	7	-	30	-	
0.625	-0.204	10	6	-	40	-	
1.25	0.097	10	5	-	50	-	
2.50	0.398	10	3	10	70	0	
5	0.699	10	2	9	80	10	*Stephania japonica* Extract (31.74)
10	1.000	10	1	8	90	20	
20	1.301	10	0	6	100	40	
40	1.602	10	0	5	100	50	
80	1.903	10	0	2	100	70	
160	2.204	10	0	1	100	90	

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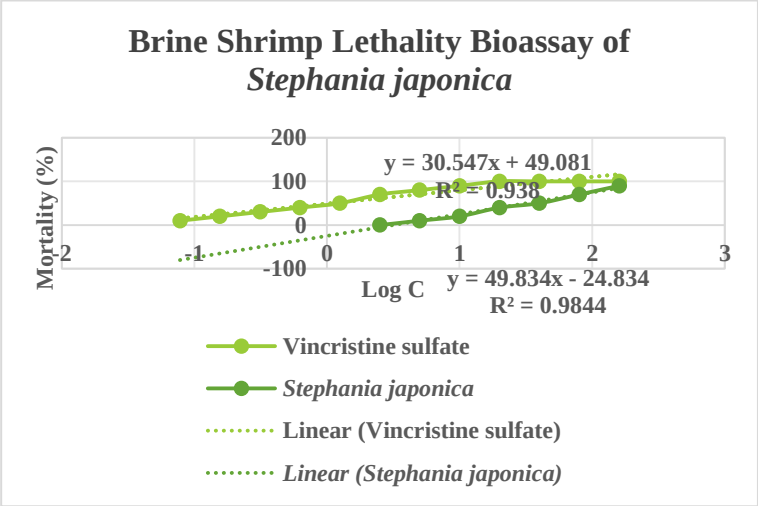

Figure 1. Comparative percentage mortality by ethanolic leaves extract of *Stephania japonica* and Vincristine Sulfate.

4.2. Antidiabetic Activity

The antihyperglycemic effect of the ethanolic leaf extract was studied using the OGTT in glucose-loaded mice. The results shown in Table 2, Figure 2, and Figure 3 show that the extract significantly decreased plasma glucose levels in the course of experiment. In particular, the extract at a dose of 500 mg/kg led to 75.13% decrease in plasma glucose level after 240 minutes while the standard drug metformin resulted in 84.41% decrease.

Table 2. Effect of ethanolic leaves extract of *Stephania japonica* on plasma glucose levels in glucose-loaded mice during the Oral Glucose Tolerance Test (OGTT).

Group	Dose	0 min	60 min	120 min	180 min	240 min	% lessening of Plasma glucose level
Control (Water)	10 mL/kg	21.32	20.02	19.34	18.58	18.08	
Standard (Metformin)	100 mg/kg	26.32	21.68	17.82	14.58	8.74	84.41
Stephania japonica Extract	500 mg/kg	26.5	24.76	21.2	17.8	15.88	75.13

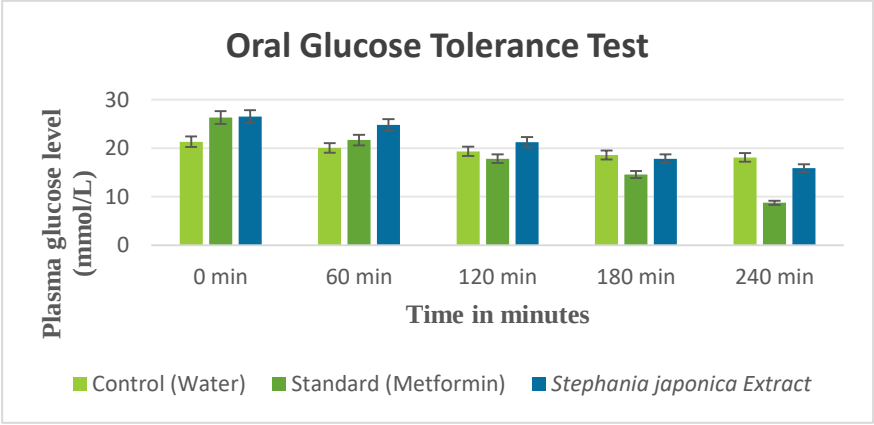

Figure 2. Effect of ethanolic leaves extract of *Stephania japonica* on plasma glucose levels during the Oral Glucose Tolerance Test (OGTT) in mice.

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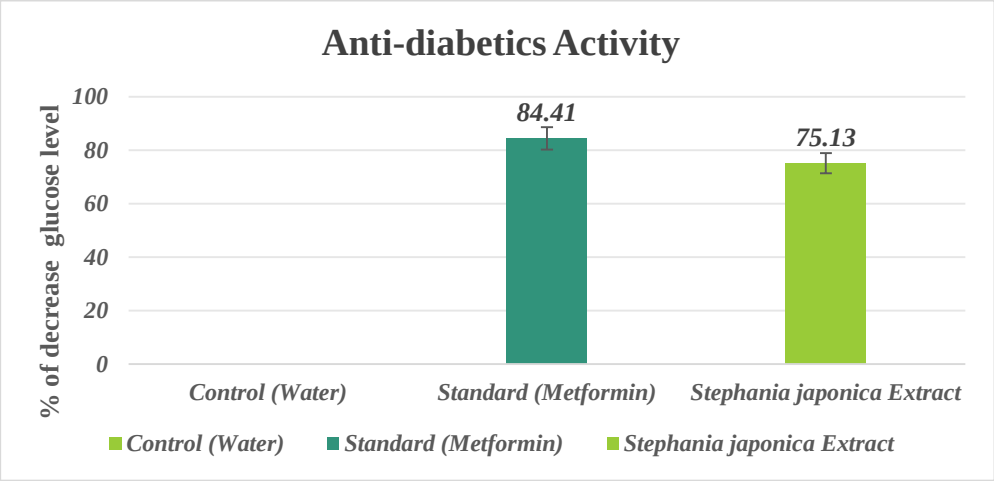

Figure 3. Comparative percentage reduction in blood glucose levels by ethanolic leaves extract of *Stephania japonica* and metformin.

The observed decrease in the blood glucose levels of the treated group shows that the extract has a pronounced anti-hyperglycemic effect. While this effect is less pronounced than in case of metformin treatment, the extract showed a steady glucose lowering effect during the entire study. This effect is presumably caused by the presence of alkaloids, flavonoids, and phenolic compounds which were proved to increase insulin secretion, promote peripheral glucose utilization, prevent glucose absorption in the intestine, and reduce oxidative stress. These findings are in line with earlier publications on the anti-diabetic effects of various species of genus Stephania and other medicinal plants with high concentration of alkaloids. Due to the fact that oxidative stress and disturbed glucose metabolism are among the primary causes of diabetes mellitus, the active ingredients of the studied plant can help to maintain glucose metabolism via various pathways. Thus, the extract can serve as a promising natural source of new antidiabetic drugs [14].

4.3. Analgesic Activity

The analgesic activity of the ethanolic leaf extract was studied using the acetic acid-induced writhing test in Swiss albino mice. As it is seen from Table 3 and Figure 4, the extract significantly decreased the number of writhes in comparison with the control group. In particular, the extract-treated group had 26 writhes with 42.22% writhing inhibition, calculated using Equation 2, while diclofenac sodium resulted in only 4 writhes and 91.11% writhing inhibition.

Table 3. Effect of the ethanolic leaves extract of *Stephania japonica* on acetic acid-induced writhing in swiss albino mice (n = 5).

Group	Dose	Number of Writhing	% Writhing Inhibition
Control	10 mL/kg	45	0
Standard (Diclofenac Na)	100 mg/kg	04	91.11
Stephania japonica Extract	500 mg/kg	26	42.22

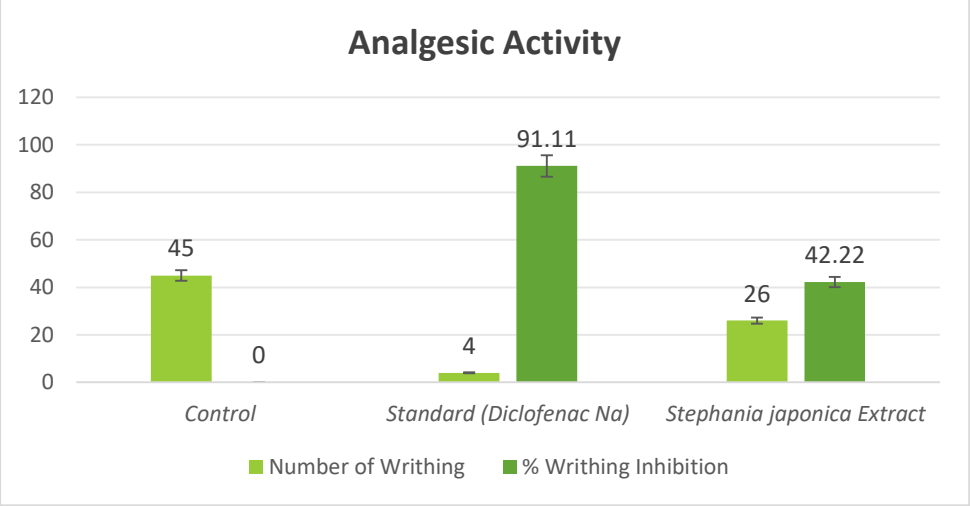

Figure 4. Analgesic activity of the ethanolic leaves extract of *Stephania japonica* in the acetic acid-induced writhing test.

Writhing response is induced by acetic acid through the release of prostaglandins, histamines, serotonin, and other inflammatory mediators. The inhibition of writhing responses in extract-treated animals shows that the ethanolic leaf extract of the plant is a peripheral analgesic. Even though the effect is not as much as that of diclofenac sodium, the inhibition implies that the plant contains components with analgesic activities. The analgesic property could be due to the presence of flavonoids, alkaloids, tannins, and phenolic compounds in the plant. The above phytoconstituents are known to inhibit the cyclooxygenase pathway, leading to a decrease in inflammatory mediator production and hence nociception. The analgesic properties have been reported in various medicinal plants from the Menispermaceae family [15].

4.4. Overall Pharmacological Significance

The pharmacological effects analyzed in the current study have shown that the ethanolic leaf extract of *Stephania japonica* has significant cytotoxic, antidiabetic, and analgesic activities. Of the three pharmacological properties studied, the highest activity was recorded in the antidiabetic test where the extract produced significant decreases in plasma glucose levels. Cytotoxic and analgesic tests were also associated with significant biological activities.

5.0. Conclusion and Further Studies

5.1. Conclusion

From the above discussion, it is evident that the ethanolic leaf extract of *Stephania japonica* shows considerable pharmacological activities that include cytotoxic, antidiabetic, and analgesic activities. According to the results obtained from the brine shrimp lethality assay, there was a concentration-dependent cytotoxic activity of the extract with an LC_{50} of 31.74 µg/mL, thus revealing the existence of biologically active substances that have cytotoxic potentials. Again, the extract showed a remarkable antidiabetic activity in OGTT where it reduced plasma glucose levels in glucose-loaded mice by 75.13% as compared to standard drug metformin. In addition, the extract showed moderate analgesic activity in acetic acid-induced writhing test where the inhibition percentage was 42.22% as compared to standard drug diclofenac sodium. The findings from this study reveal that *Stephania*

japonica is a highly promising medicinal plant with good pharmacological activities. The activities can be attributed to the existence of various bioactive phytochemicals such as alkaloids, flavonoids, phenolic compounds, tannins, and glycosides.

5.2. Future Recommendations

Despite the promising pharmacological activities identified in the present study of ethanolic leaf extract of *Stephania japonica*, more studies are required to elucidate the full range of its medical potential. In particular, future studies should address:

1. Isolation, purification, and determination of structure of active compounds responsible for the pharmacological activity of the extract.
2. Clarification of the mechanism by which the cytotoxic, antihyperglycemic, and analgesic properties of the extract operate.
3. Determination of dose response relationship and determination of the optimum therapeutic dose of the extract.
4. Antihyperglycemic activity assessment of the extract using alloxan or streptozotocin-induced diabetic animals.
5. Determination of anti-cancer potential of cytotoxic fractions.
6. Estimation of toxicity of the extract by conducting appropriate toxicological studies.
7. Study of other pharmacological activities which might underlie the observed effect.
8. Clinical investigation of efficacy and safety of *Stephania japonica* if preclinical studies indicate promising results.
9. *Stephania japonica* is a unique medicinal plant with great potential for various pharmaceutical and therapeutic applications. Comprehensive research will allow for creation of new medicines derived from this plant.

Declarations

Source of Funding

This study received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Competing Interests Statement

The authors declare that they have no competing interests related to this work.

Consent for Publication

The authors declare that they consented to the publication of this study.

Authors' Contributions

Md. Mahbubol Alam, Mohaiminul Islam and Mosa. Tanha Aktar took part in literature review, conceptualization, methodology, investigation, analysis, and manuscript writing and editing. Md. Mahbubol Alam, Fahamida Binta Anower and Ahsan Ullah analyzed data. Nowrin Rashid Ratri, and Durdana Abthahi were involved in the design

of the manuscript. Ahsan Ullah and Fahamida Binta Anower reviewed the manuscript. All authors have read and approved the final manuscript.

Availability of Data and Materials

Supplementary information is available from the authors upon reasonable request.

Institutional Review Board Statement

Not applicable.

Ethical Approval

All animal experiments were conducted in accordance with the institutional guidelines for the care and use of laboratory animals. Every effort was made to ensure the humane handling of animals and to minimize pain, discomfort, and stress throughout the experimental procedures.

Informed Consent

Not applicable for this study.

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Declaration of Artificial Intelligence

No AI tools were used for this study.

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